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Uncovering Candidate Cold Tolerance Genes in Maize (Zea Mays)

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Uncovering Candidate Cold Tolerance Genes in Maize (*Zea Mays*)

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Contents:

Abstract

With booming populations soon to overwhelm the world's food production capabilities, studying what makes crop organisms, like maize, efficient is crucial to ensure that the demand for food is met. Planting earlier in the spring would lengthen the crop season and produce larger yields provided the crop is tolerant to early spring's low temperatures. Plants can adjust to abiotic stresses through biochemical changes controlled by transcription of genes. Trained plants can be produced by pre-exposing them to a lesser stress, allowing them to recover, then exposing them to a greater, longer stress. It is hypothesized that trained plants will tolerate the second harder stress more than untrained plants. There are also many different lines of maize with varying degrees of cold tolerance regardless of training. For instance, B73 is known to be less cold tolerant than Mo17.

In this study, candidate cold tolerance genes were identified using RNAseq of maize seedlings exposed to multiple cold stresses. Quantitative trait loci analysis were conducted on 95 lines of the IBM population and their parents (B73 and Mo17). These analyses elucidated the differences in cold response between initial and repeated cold exposure as well as the difference between cold susceptible B73 and cold tolerant Mo17 maize lines.

Through support of phenotypic measurements (total height, leaf greenness, chlorophyll concentration, and a 3-point visual scale), it was concluded that previous cold exposures offered no benefit to the maize seedlings after stress. Four QTL regions on chromosomes 1, 3, 5 and 7 were well supported with combinations of the phenotype data. Differential expression data from cold stress experiments were used to identify 13 significantly differentially expressed genes within the QTL regions and identify 20 candidate cold memory genes by comparing transcriptomes of trained and untrained plants. These genes potentially explain the difference in response to initial versus secondary cold exposures and cold stress response between B73 and Mo17.

Introduction

Maize is the most widely distributed crop in the world with approximately 90 million acres planted in the United States every year (Kumar, 2013; United States Department of Agriculture, 2016). Its presence in our daily life is undeniable; besides covering nearly 30% of all crop acreage in the United States, it powers the economy by producing exports, livestock feed, ethanol fuel, and food worth over 65 million dollars (National Corn Growers Association, 2015). In developing countries, corn is grown less commercially, usually by poor farmers using their harvests as their primary food source. In Africa, for example, two-thirds of the produced corn is used for human consumption (Shiferaw, 2011). In the world, maize has elevated its status from plant to commodity: it is grown for many purposes, through trade or direct consumption, it supports human vitality worldwide.

In the last 100 years, the global population has increased by six billion people and is expected to increase at a rate of one billion people per decade until stabilizing at a population of 10 billion (Borlaug, 2002). With this increase in mouths to feed, global demand for crops like maize are sure to increase. Demand for corn is especially growing in countries like China and India where their economic growth is allowing many to afford eggs and meat (Shiferaw, 2011). The availability of these animal products relies heavily on the maize market as corn is a popular livestock feed. The capability of the maize market to keep up with this increased demand is unlikely without drastic change.

The high yield and versatility of maize makes it a desirable crop for farmers around the world; It represents 27% of the all cereal growth area but 34% of cereal production (Shiferaw, 2011). This high productivity on even less land area makes maize an attractive crop. It also contains a higher protein and fat content than other cereal crops (Kumar, 2013) and is capable of

being bio fortified to increase nutritional content (Shiferaw, 2011). Beyond its nutritional content and efficiency, maize can grow in a greater variety of environmental conditions than any other crop (Shiferaw, 2011). If any crop is capable of feeding the booming global population, it's maize.

While global population growth is increasing demand for maize, it is also hampering its production as climate change negatively impacts fields across the globe. Increasing population is also increasing pollution in the soils, air and water. These changing components of the surrounding environment are creating abiotic stresses for plants and crops alike. Plants are completely sessile; they must cope with all stresses they encounter because escaping is not an option. Thus, plants have developed many biochemical mechanisms to tolerate stresses such as synthesizing toxins, ceasing growth and managing salt concentration (Shanker and Venkateswarlu, 2011; Farooq, 2009). All stresses typically limit the productivity of crop plants.

Temperature variation, resulting from climate change, is a threat to maize that is vital to our everyday economy and lifestyle. Only one-third of the total land mass is free of ice and 42% regularly experiences temperatures below 20 degrees Celsius (Janska, 2010). Temperatures below 20 degrees Celsius are known to strongly limit growth and development of maize (Sobkowiak, 2016). Low temperature stress has multiple negative effects on plant function causing slowed growth, photosynthesis cessation, biochemical changes, tissue damage, and if severe, death (Shanker and Venkateswarlu, 2011). Maize is largely considered a cold sensitive crop due to its origins in the tropics. Domesticated from teosinte by Aztec people in the central Americas, maize has recently been impacted with limited germination and production levels by consistently colder temperatures in early spring (Hu et. al, 2016). Cold stress is one of the most

harmful abiotic stresses affecting maize (Janska, 2010). It affects nearly all aspects of the cell eliciting a cellular response on all levels (Chinnusamy, 2007).

In response to cold, plants have evolved mechanisms to cope with this stress by making physiological adjustments that are likely controlled by changes in gene expression. Most commonly, the membrane changes composition to include more or less phospholipids and sugars (Mickelbart, 2015; Thomashow, 1999; Viswanathan, 2007; Janska, 2010; Sanghera, 2011; Ding, 2014) and plant cells accumulate antioxidants to battle reactive oxygen species (Chinnusamy, 2007; Janska, 2010; Micklebart, 2015; Sobkowiak, 2014). There are reports of observing global patterns of gene expression induction in gene ontology (GO) categories such as: DNA conformation change, translation, protein refolding and intracellular import (Sobkowiak, 2016; Chinnusamy, 2007; Ding, 2014). Contrasting results of global repression of gene expression include GO categories scvh as: cell redox homeostasis, photosynthesis, and regulation of macromolecular biosynthesis (Sobkowiak, 2014). Many researchers agree that response to cold stress is complex with many levels of gene regulation. Induction of transcription factor genes suggests a coordinated response from many signaling pathways (Ding, 2014; Frey, 2015; Sobkowiak, 2016; Mao, 2016; Chinnusamy, 2007). Complexity of the basis of physiological resistance to cold impedes developing cold resistant maize varieties through traditional breeding programs.

Much of the maize grown today is cultivated far outside of its original geographical region in environments that do not reflect its primitive zone of natural selection (Greaves, 1996). Many of these regions have expanded to the far north, into Canada, northern Midwest, and northern Europe where the temperatures regularly fall below optimal temperatures. The strategies for coping with this regular cold stress varies in different northern lines. Some increase

stomatal resistance or increase water uptake with large and deep root systems. Others accumulate osmolytes, osmoprotectants, or antioxidants within their cells. Another strategy employed by maize in response to cold is to stabilize the cell membrane or express aquaporins (Farooq, 2009; Mickelbart, 2015; Thomashow, 1999; Viswanathan, 2007; Janska, 2010; Sanghera, 2011; Ding, 2014). Realistically, a combination of these molecular strategies is employed to confer cold tolerance in a variety of ways.

To better respond to future climatic changes, it is important to develop a maize line with greater cold tolerance. Modern breeding programs target molecular markers within the 2.5 billion base pair maize genome to identify genome loci that carry genes responsible for cold tolerance and increase the precision of plant breeding (Collard, 2008). This process starts with quantitative trait locus analysis (QTL) where the co-segregation of variation in cold stress response is analyzed in recombinant inbred lines. The QTL analysis identifies regions of genome potentially responsible for the trait. This approach could be augmented by analyzing whole genome gene expression levels in response to stress to find genes activated in response to stress.

The goals of this project were to (I) develop quantitative assays to measure cold stress response in maize seedlings, (II) characterize the phenotypic response to, and global gene expression as the result of repeated stress exposure, and (III) identify candidate cold tolerance genes within the maize genome using QTL analysis. The cold tolerance genes identified could become promising candidate genes for future mutant analysis. Expression analyses can only speculate the genes effect on phenotype thus, direct effect must be confirmed through silencing or overexpression of the candidate genes. Once the functional mechanisms of the candidate genes can be validated, the understanding of cold tolerance as a complex phenotype can be better understood and perhaps utilized in modern breeding programs.

Part I of this thesis describes a cold stress priming experiment. The type of cold stress and the age of the plant at the time of cold stress was manipulated to investigate the phenotypic response and gene expression changes as the result of repeated stress exposure. Part II describes the natural variation in seedling exposure to cold stress in two lines: B73, known to be cold susceptible and Mo17, a cold tolerant variety. A quantitative trait locus analysis was conducted using intermated recombinant inbred lines (RILs) and the parents (B73 and Mo17) to identify genomic regions where candidate genes, potentially conferring cold stress tolerance in maize seedlings, localize.

Part I: Priming of Cold Stress

Background

Stress response in maize is controlled by a complicated system of pathways linking signals from the environment to regulatory features that alter the expression of genes. This response is known to vary among different inbred lines and in response to various abiotic stresses, such as cold, heat, drought, or high salinity (Waters, 2016). The regulatory features that respond to abiotic stress can take several forms including activation of transcription factors (TFs), silencers, enhancers and transposable elements (TEs). These TEs have regulatory sequences that can affect the regulation of adjacent genes in response to environmental stress (Makarevitch, 2015). Within such a complicated system of regulation, it is possible that an underlying mechanism exists to retain stress induced changes ensuring better preparation for the next stress event.

Stress acclimation is defined as increased tolerance to a severe stress through exposure to a weaker stress (Sobkowiak, 2016). This phenomenon has been observed in several plant species; alfalfa and canola can acclimate to cold (Chinnusamy, 2007), while arabidopsis and maize have been shown to acclimate to drought (Ding, 2014), and cold (Sobkowiak, 2016). The environmental stress is hypothesized to signal complex cellular activity that changes regulation of many stress response genes (Chinnusamy, 2007). Different transcription patterns have been observed in response to an initial stress versus a second stress (Ding, 2014), possibly allowing the plant to prepare for repeated stresses and improve recovery and chances of survival.

Cold stress primarily injures the membranes within plant cells (Thomashow, 1999; Gulzar, 2011). Many biochemical changes may occur to stabilize the membrane during cold

acclimation. For instance, changing the composition to include different phospholipids or sugars is very successful in eliciting cold tolerance (Chinnusamy, 2007; Janska, 2010; Farooq, 2009; Mickelbart, 2015; Ding, 2014). Accumulating antioxidants (Mickelbart, 2015; Sobkowiak, 2014; Farooq, 2009) or osmoprotectants (Mickelbart, 2015; Farooq, 2009) have been shown to confer tolerance to cold stress as well. The increased rigidity of the membrane together with osmoprotectants and antioxidants in high abundance allows the plant to be prepared for additional stresses in the near future.

The effects of cold acclimation were first observed in hardwood trees which tolerate harsh winters on a yearly basis. This extreme tolerance is known to exist in most temperate plants up to a point of ice formation within their vegetative tissues (Chinnusamy, 2007). While the effects of cold acclimation within hardwood trees are some of the most dramatic, cold acclimation does occur to varying extents in other plant species. While maize has been shown to acclimate to other abiotic stresses, it is less known if maize could be primed to cold stress in a laboratory setting and if such priming could have functional significance.

There are discrepancies in the literature as to whether maize is capable of cold acclimation. It is frequently described as both a tropical and temperate plant due to its incredible range throughout the world (Shiferaw, 2011). However, accounts of maize cold acclimating are present. Sobkowiak's research in 2016 found that cold acclimation in maize only marginally improves cold tolerance, but is necessary to activate the cold resistance of cold tolerant maize lines. The temperature threshold can only be lowered a few degrees Celsius as maize does not resemble the frost tolerance of plants like arabidopsis. However, using a four day 14/12 degree Celsius priming followed by a four day 8/6 degree Celsius stress improved cold tolerance in maize. Farooq's research in 2009 also showed that maize can be acclimated to cold stress with a

brief 4 degree Celsius chilling stress. Chinnusamy admits in 2007 that low temperature induced changes can be observed even in chilling-sensitive crops by prior exposures to suboptimal temperatures.

In this project we hypothesized that maize seedlings could be acclimated to cold by repeated exposure to cold stress. We analyzed the phenotypic response to cold using an image analysis and chlorophyll concentrations to quantify greenness and infer general leaf health. Gene expression analysis was used to identify changes in response to an initial stress and repeated stresses and select genes that could be important for acclimation.

We expected to observe maize acclimation to cold through a training regime using four treatment groups that were monitored throughout their growth. Control plants grew in optimal conditions, stressed plants were exposed to a hard stress (4 degrees Celsius) at the end of the 16 day experiment, primed plants were only exposed to the early, mild acclamatory stress (6 degrees Celsius) on the 8^{th} day and finally primed and stressed plants were exposed to both types of cold stress. All plants were measured for total height every day by measuring from the top of the potting container to the tip of the tallest leaf. At the end of the last hard stress, plants were sacrificed to conduct an image analysis on the third leaf then obtain chlorophyll concentrations. These measurements were expected to capture the effect of cold stress often described as burning. Usually, the tips and edges of the leaves become dehydrated and brown. The image analysis was sensitive to these defined burn marks where the chlorophyll concentration was found to have greater sensitivity to paling leaves. We hypothesized that the primed and stressed treatment group would outperform the stressed treatment group due to the regulatory changes experienced during the priming exposure.

In addition to the phenotypic analysis of priming maize in response to cold stress, three plants from each treatment group were sacrificed two hours into the final hard stress. Global gene expression profiles were obtained by RNAseq analysis to find genes potentially responsible for cold memory. The genes identified are hypothesized to be candidate cold tolerance genes that influence the seedling's ability to resist multiple cold stresses.

Materials and Methods

Training Regime

B73 maize seedlings were grown in 1000 cm^3 containers with all-purpose growing soil mix. These plants were watered regularly while exposed to a priming and stressing regime using Conviron cabinet growth chambers diagramed in Figure 1. Priming exposure was designed to avoid stunting seedling growth where the stressing period was intended to have a harsher effect on seedlings.

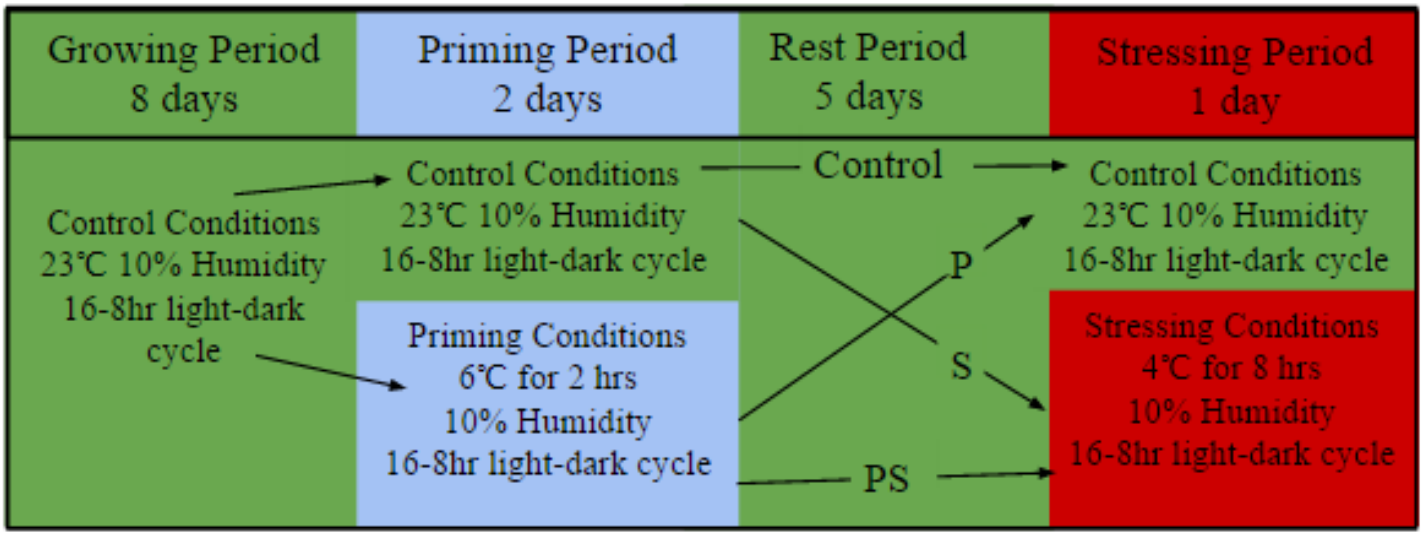


Figure 1: Control, primed (P), stressed (S) and primed and stressed (PS) treatment group exposure timelines. Growth measurements were collected throughout the training regime. Phenotypic analysis occurred 2 days post stress. RNAseq samples were collected from the 3rd leaf of all treatment groups 2 hours into the final stressing period.

Growth Assay

Growth was measured daily as total height in centimeters. A rigid piece of paper was laid across the container to ensure the same base was used for each measurement. The plant was then measured from the paper base to the tallest leaf by lifting the leaves as seen in Figure 2.

Figure 2: Example of seedling total height measurement with paper base and lifted leaves.

Image Analysis

Leaves were wiped with deionized water to remove any marks or debris before being imaged flat with blade down using an Epson scanner with a contrasting paper background. Image analysis was conducted using a Mathematica function called Dominant Colors. This function breaks an image into a specified number of color clusters, usually capturing any yellowing or burning that resulted from cold stress seen in Figure 3. The areas of these color clusters were then compared to the area of the whole leaf to get a percentage of "purple" vein and "gray"

stressed region. The remaining portion of the leaf was assumed to be heathy “green” tissue. This method quantified the unique burning patterns observed on individual leaves.

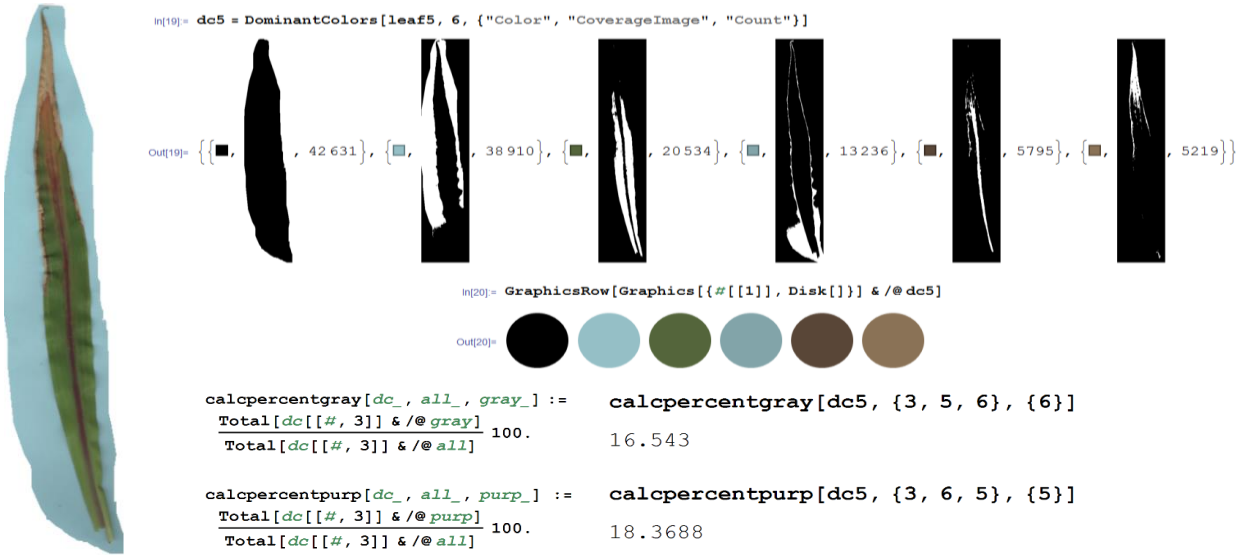


Figure 3: Raw outputs of Image analysis within Mathematica. Formula definitions and calls are shown as well as Dominant colors output with GraphicsRow output to better depict color. The number of Color Clusters could be increased to generate greater sensitivity to smaller areas of burned tissue.

Chlorophyll Concentration Assay

Chlorophyll analysis was conducted using a methanol solution and absorption method from Michigan State University. One half gram of leaf tissue was collected from the tip of the third leaf then ground to a fine powder with liquid nitrogen. The leaf powder was combined with 15 mL methanol and filtered into a cuvette. Figure 4 shows the wide variety of solution color collected from stressed and healthy leaves. Methanol was used as a blank for reads at 663nm (Chlorophyll A) and 645nm (Chlorophyll B). The formulas in Figure 5 are derived from Beer’s law to calculate the concentration of chlorophyll per gram of sample.

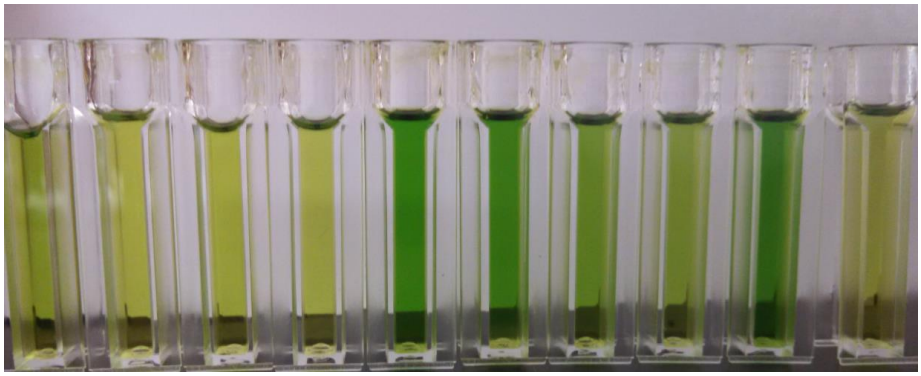


Figure 4: Variation in color of filtered methanol and plant tissue in cuvettes.

$$\text{Chlorophyll A (mmol/L)} = \frac{\text{Abs at 663 nm}}{75.05\ L/\text{mmmol–cm} * 1.17\ \text{cm}}$$

$$\text{Chlorophyll B (mmol/L)} = \frac{\text{Abs at 645 nm}}{47.0\ L/\text{mmmol–cm} * 1.17\ \text{cm}}$$

Figure 5: Equations derived from Beer's Law to calculate chlorophyll concentration from Michigan State University's chlorophyll extraction method.

RNAseq Analysis

RNA samples were collected from the third leaf of each seedling and RNA was extracted by TRIzol RNA isolation procedure according to manufacturer's instructions. Eight plants were combined for each of the three replicates of four experimental conditions. RNAseq analysis was conducted in the University of Minnesota Genome Center for these twelve samples and aligned to the B73 maize reference genome. RNAseq is a highly sensitive global gene expression analysis where mRNA collected from the organism is converted into cDNA. Gene expression is quantified by measuring the number of times sample cDNA fragments align with a template DNA strand for each gene. These counts were normalized by gene length and analyzed using DEseq in R (Anders, 2010) to find differentially expressed genes that have a p-value less than

0.05. This provides an accurate measurement of gene expression for the entire genome and allows identification of genes that are affected by cold stress.

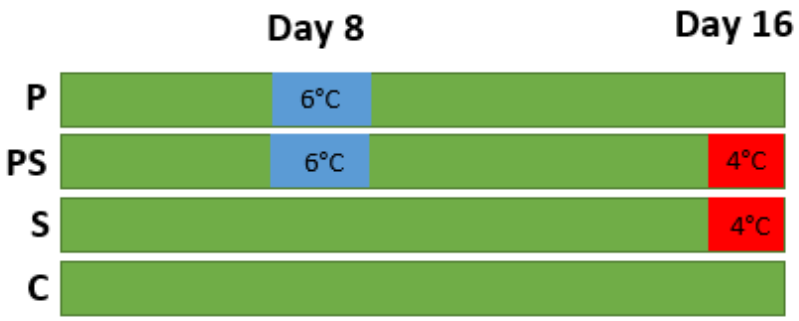


Figure 6: Priming treatment groups sent for RNAseq analysis in replicates of three. All plants were 16 day old B73 seedlings with tissue collected from the third leaf after experiencing 2 hours of final stress.

Results

B73 maize seedlings were exposed to a training regime to cold stress as described in Figure 1. Each treatment was grown in replicates of 6 seedlings and exposed to the same environments within Conviron growth cabinets. To quantify the effects of treatments on seedlings, plant growth was monitored throughout the study and the state of the seedlings was recorded after final cold stress using image analyses and chlorophyll concentration.

Heights of maize seedlings were recorded daily throughout the training regime. The measurements were averaged to reveal that early priming events were not severe enough to slow growth while later stressing events slowed growth temporarily. Initial measurements showed a recovery trend that suggested trained plants were regaining height faster than untrained plants (Fig. 7A). To confirm this trend, plant's height was measured each day for longer after the stressing date to better examine the recovery period (Fig. 7B). The increase in recovery of the trained plants wasn't maintained in the second trial. The trained plants continued to behave

similarly to the untrained plants suggesting the cold training regime had no beneficial or negative impact upon growth rate of maize seedlings.

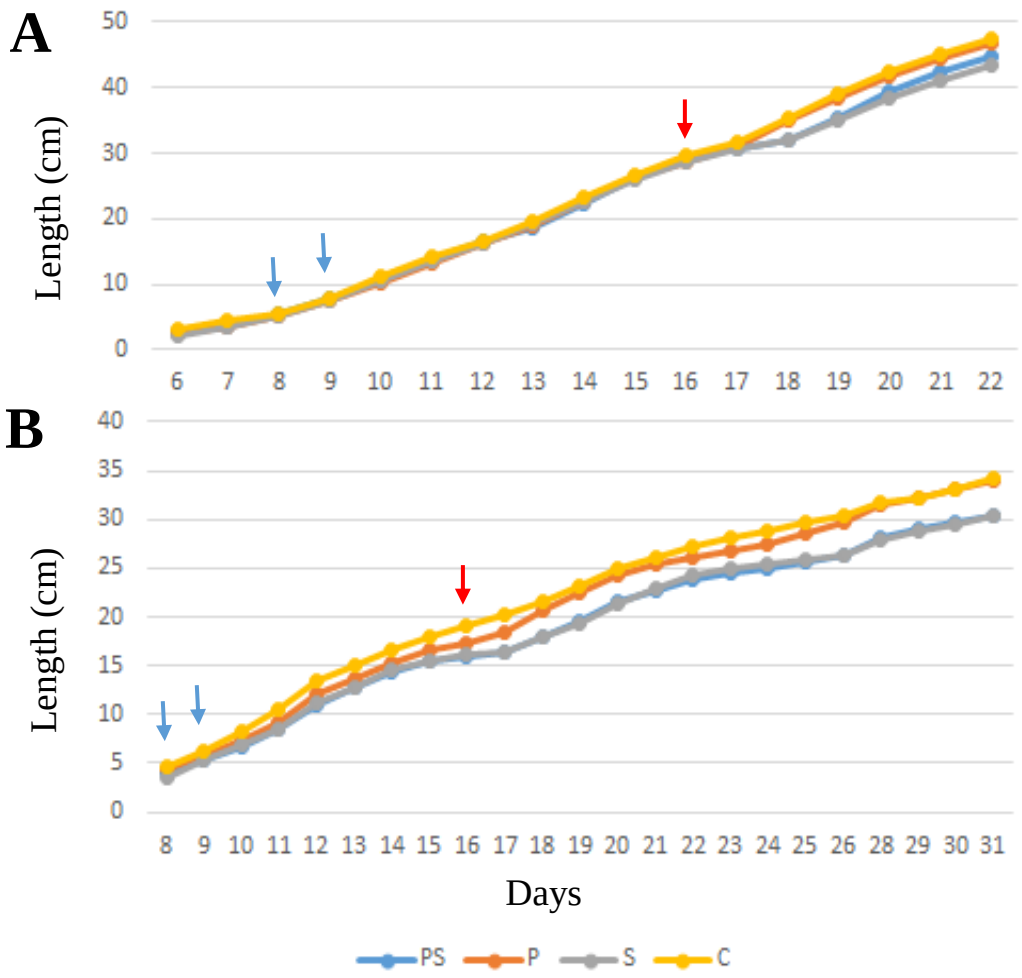


Figure 7: Total height measured in centimeters plotted by days of growth. Blue arrows mark days of 4 hour 6°C priming and red arrows mark the day of 8 hour 4°C cold stress. **A)** Early growth trials suggesting quicker recovery for the trained treatment group after stressing when compared to the untrained treatment group. This is seen as the PS treatment group nearing heights comparable to the control at day 20, where the S treatment group remains stunted in growth. **B)** However, this second trial shows this suspected recovery trend was not present weeks after the stress date. Trained plants (PS) grow similarly to untrained plants (S).

Burning of leaf edges is a common consequence of cold stress in maize seedlings. These patterns of damage can easily be used to visually determine the degree of stress a plant has

experienced. In the past, number scales were used to assign degrees of stress to plants. However, this isn't a reliable measurement due to the subjectivity depending on the observer. To quantify the visual damage of cold stress on seedling leaves and remove the subjectivity, an image analysis method was developed. Images of leaves were collected and used to measure pixels of "damage" (gray) as a percentage of whole "leaf" pixels. Because plants are not visually damaged immediately following stress, images were collected 2-3 days post stress exposure to allow plants to develop burnt edges for measurement.

It should be noted that an unexpected purple vein also developed as the plants grew. This vein varied in thickness and depth of color. It was hypothesized that the degree of "purpling" would increase upon stress exposure despite being present in all plants. The image analysis was flexible enough to account for several colors on each leaf and thus percent purple was added to the analysis.

The average percentage of each color for each treatment varied greatly. No significant differences were found even when comparing the control to the stress group (Fig. 8). However, when comparing proportions of damage to purpling, the primed treatment group responded to the stress similarly to the control group, suggesting the recovery from the original stress, and the trained and untrained plants behaved similarly to each other, suggesting that priming provided no benefit for the trained plants in response to cold. It was also found that purpling did not worsen in response to cold stress but possibly in response to greater growth. The primed and control groups grew to greater heights than the other two treatment groups and experienced the greatest purpling of their leaves' vein.

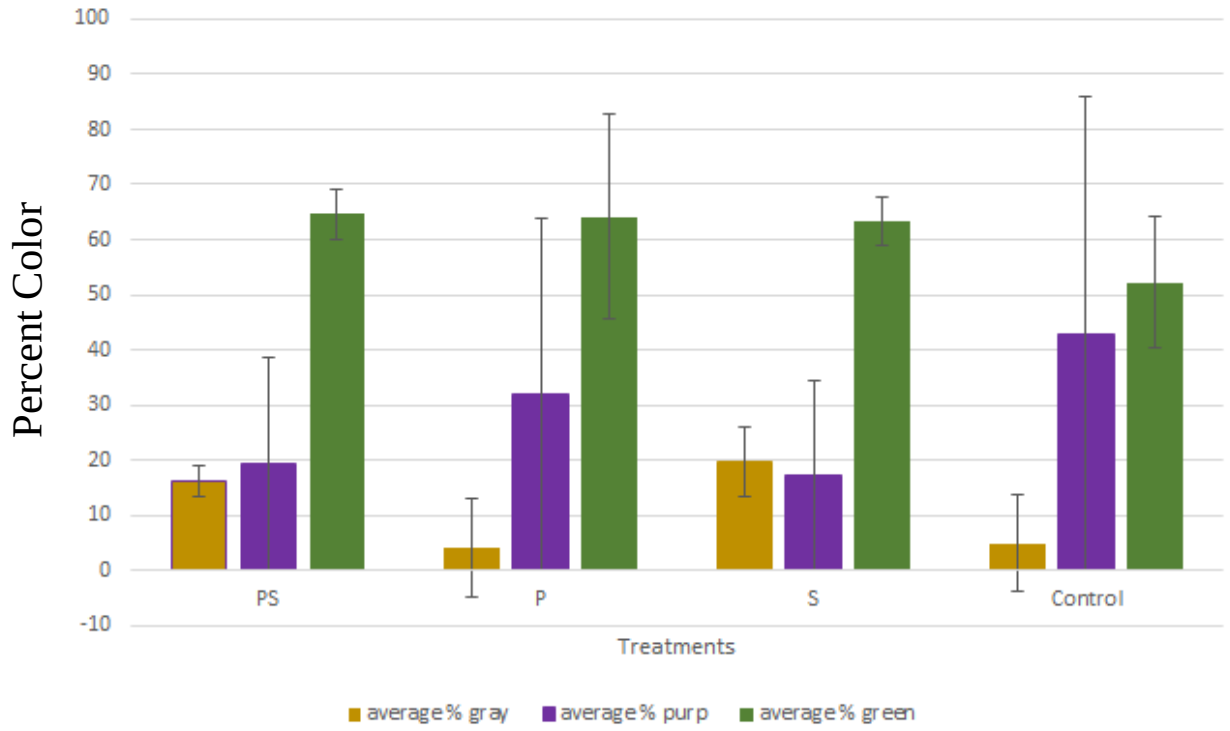


Figure 8: The percentage of damaged, purple, or healthy tissue found on the 3rd leaf of 18 day old maize seedlings by treatment group. Image Analysis suggests trained plants (PS) respond most like stressed plants (S), while primed plants (P) respond most like the control group. This suggests that priming did not have a lasting effect on the plant's stress response. Error bars show standard deviation of each measurement.

Chlorophyll concentrations have been known to change in response to abiotic stress such as cold stress. It can be observed with the naked eye that plant leaves become less green in response to suboptimal temperatures. Chlorophyll concentration is a relatively simple way to measure the damage endured during cold stress. Again, no significant results were found between any of the treatment groups. All treatment groups exposed to cold conditions had lowered chlorophyll A concentrations (Fig. 9). The priming treatment group has maintained its proportion of chlorophyll A to chlorophyll B where the trained and untrained treatment groups have more chlorophyll B in comparison to the levels of chlorophyll A. This is unexpected as Chlorophyll A has been described as participating in resistance to low temperature (Babenko, 2014).

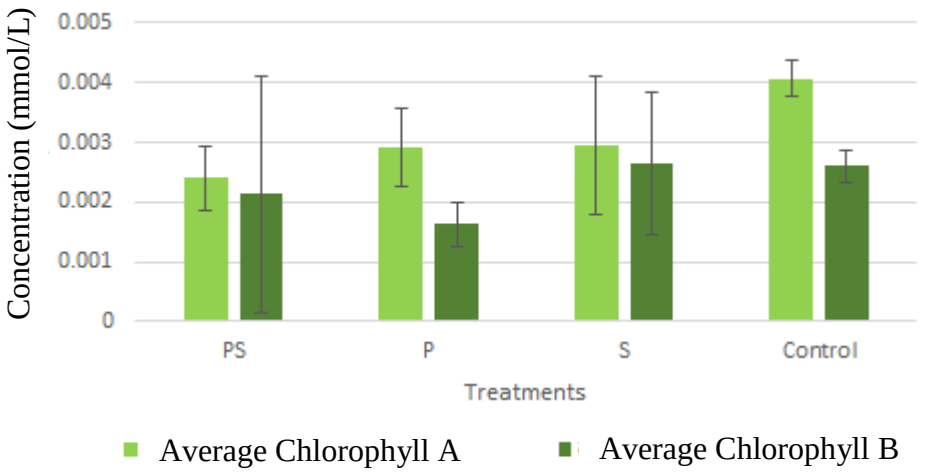


Figure 9: Concentration of chlorophyll in mmol/L found in the 3rd leaf of 18 day old maize seedlings by treatment group. Chlorophyll concentrations in trained plants (PS) are most similar to concentrations in stressed plants (S) based on the proportion of Chlorophyll A and B. Error bars show standard deviation of each measurement.

In each analysis of cold tolerance phenotypes, large error bars resulted due to the great variety of response observed. It had been assumed that replicates of 6 genetically identical plants given identical treatments would have similar responses. However, a wide range of results for both image analysis (Fig. 12A) and chlorophyll concentrations (Fig. 12B) arose for each

treatment group. It became clear that larger sample sizes would be needed to calculate an accurate average for stress response in maize seedlings.

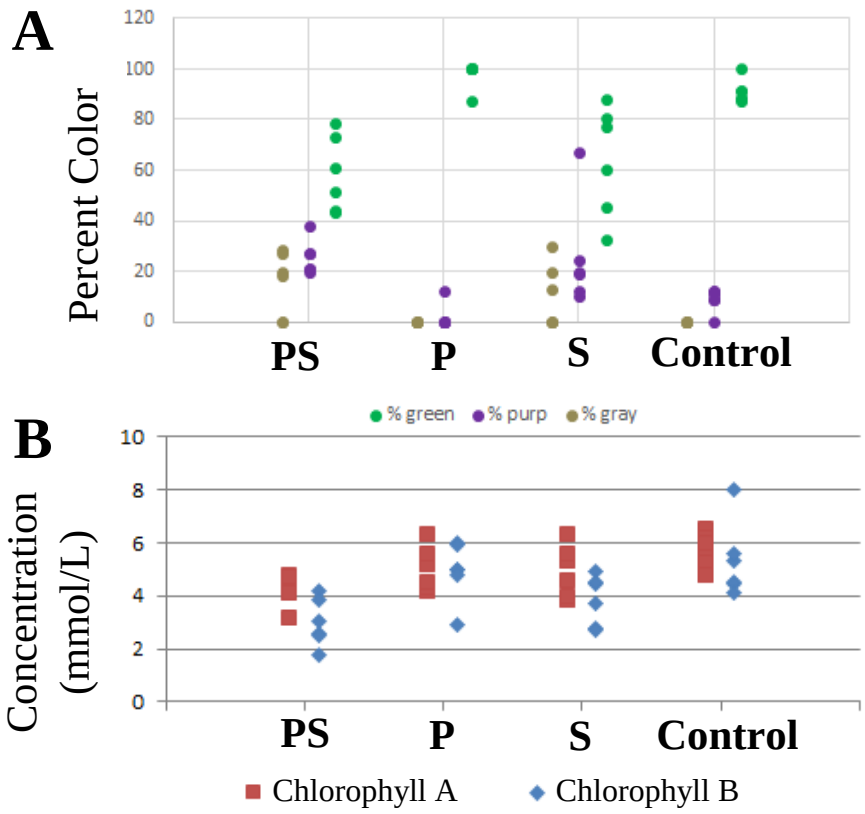


Figure 10: Dot plots of phenotypic data from individual plants to display variation. **A)** Image analysis shows each plant responds differently to stress. Some lose more "greenness" than others as seen by the large spread of data points. **B)** Chlorophyll concentration is also variable within treatment groups. This is a direct result from tissue damage variation.

Our phenotypic results didn't produce any significant results due to large variation of cold response however, our gene expression analysis produced greater success. Differentially expressed genes were identified when comparing the primed treatment group to the control. Two hundred and twenty-six genes changed in expression in response to the 6 degree priming exposure and remained changed in their expression for 8 days when tissue was collected for RNA isolation. While these genes were altered in response to cold exposure, it isn't confirmed if

they play any role in a secondary stress exposure. To evaluate this, we identified differentially expressed genes between the stressed and the primed and stressed treatment group. This identified 102 genes altered in expression when exposed to an initial cold stress versus a subsequent cold stress. All of these genes respond to cold exposure but what is remarkable, is they change the way they are expressed depending upon the history of the plant. Genes contained within both lists are very interesting. Twenty genes were found to be differentially expressed in both cases. These genes both remained changed in expression for 8 days following the priming exposure and are expressed differently in initial stress and secondary cold stresses.

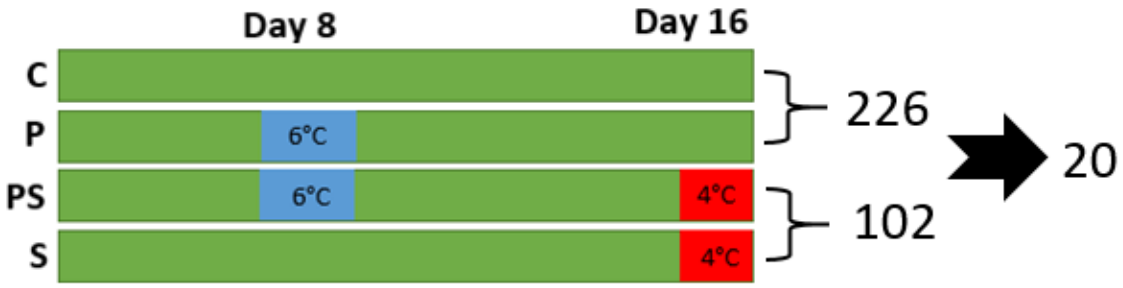


Figure 11: Method of analysis for identifying candidate genes within the priming RNAseq data. Differentially expressed genes were identified as having a fold change greater than 8 or less than 0.125. Brackets in this diagram indicate comparisons between the treatment groups revealing 226 genes between Control and primed and 102 genes between primed and stressed and stressed. The black arrow indicates a comparison of gene lists to identify genes within both lists. Twenty genes were identified within both comparisons.

Table 1: Twenty genes identified as significantly differentially expressed between Control and Priming as well as between Stressed and Primed and Stressed. Log2 fold change values are listed between each comparison. Due to the unique responsiveness of these genes, they are likely cold "memory" genes.

Gene ID	Log2 fold change in expression between		Function
	P and C	S and PS	
AC193591.3_FG001	-3.768	3.776	ORF
AC194362.3_FG003	-3.264	3.516	Protein coding, DNA binding factor
AC204763.2_FG001	3.416	-4.705	Protein coding, oxidation-reduction process
GRMZM2G005066	4.370	-3.260	c1 - colored aleurone1
GRMZM2G045155	-6.244	3.172	B12D protein
GRMZM2G076896	-3.322	3.351	AP2-EREBP-transcription factor 111
GRMZM2G088007	3.477	3.087	Protein coding, protein phosphorylation
GRMZM2G089493	-6.316	-4.284	LLA-115 (nutrient reservoir)
GRMZM2G103085	-4.223	4.100	AP2-EREBP-transcription factor 139
GRMZM2G124799	-3.229	3.206	methyltransferase
GRMZM2G148800	3.111	4.671	Protein coding, membrane transport
GRMZM2G163533	-3.714	3.557	Protein coding, lipid catabolic process
GRMZM2G178074	4.633	-3.714	phosphoenolpyruvate carboxylase kinase1
GRMZM2G339725	3.122	-3.653	Putative uncharacterized protein
GRMZM2G343291	-3.055	-4.703	Putative uncharacterized protein
GRMZM2G368610	-5.650	3.961	Phospholipase A(1)
GRMZM2G387341	-4.197	4.168	Hypothetical Protein
GRMZM2G398198	-3.507	4.178	ORF
GRMZM2G544539	-3.036	3.533	AP2-EREBP-transcription factor 28
GRMZM5G829897	-3.524	3.438	Carboxylesterase

Many of the candidate cold memory genes identified through the gene expression analysis have functions relating to gene regulation. Three transcription factors were identified where all are down regulated in both comparisons. Protein kinases and phosphorylation proteins were also identified to be differentially expressed. Finally, a methyltransferase was found to be downregulated in both comparisons. Discoveries like these support the notion that maize could

contain a cellular machinery capable of regulating the expression of stress responsive genes in a way that provides “memory”.

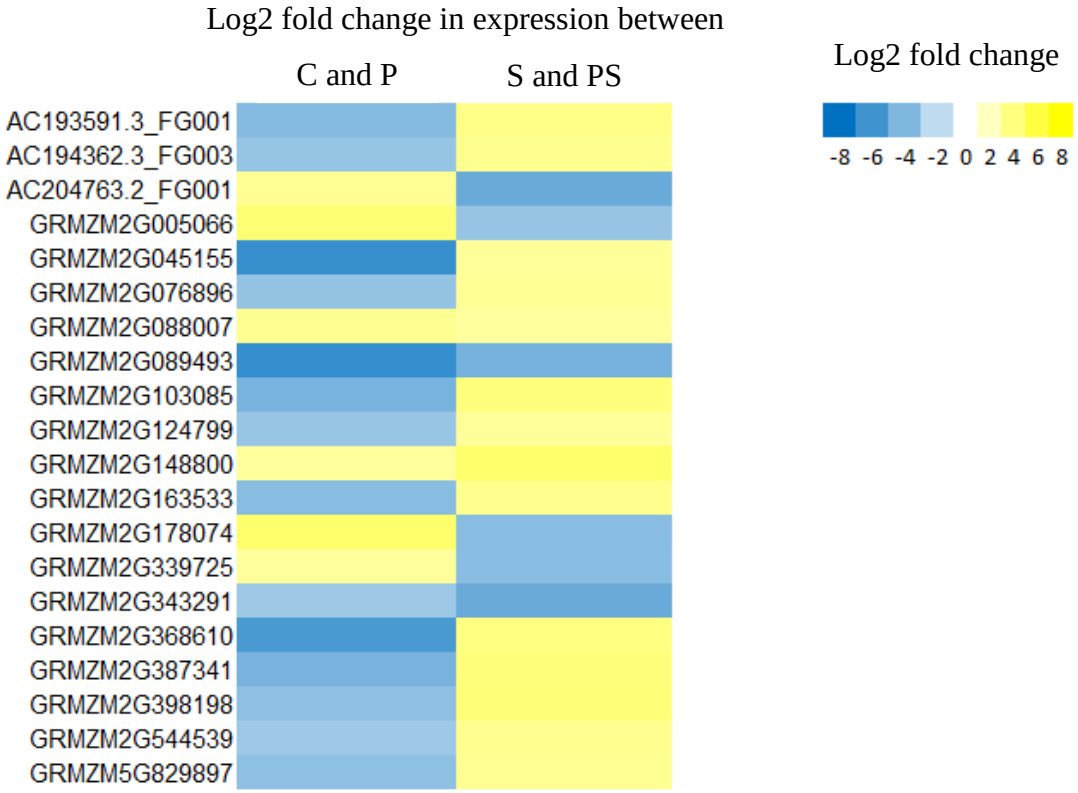


Figure 12: Heat map of log2 fold change between the treatment comparisons made to identify 20 cold memory genes. Blue shows repression where yellow shows induction of gene expression.

Surprisingly, the response of the candidate gene to an initial stress, is often the opposite of its response to a secondary stress. The left column shows induction and repression of genes in response to being exposed to the early priming stress. A majority of these genes remain downregulated until sample collection 8 days post exposure. The right column displays induction and repression of genes when experiencing a second cold stress when compared to an initial cold stress. Here, the majority is induced. The cold memory candidate genes behave as if they are activated by a second cold stress exposure.

Discussion

Immediately after stressing, trained plants resembled untrained plants visually. Image analysis and chlorophyll concentrations of trained plants also resembled plants experiencing stress for the first time. This suggests that priming had no effect on the maize seedling's response to the second stress. Otherwise, trained plants would resemble primed plants that have continuously resembled control plants throughout all analyses. It is still unknown if this is a result of error in our experiment protocol, or if maize is truly incapable of "remembering" stress. In 2014, Sobkowiak states that acclimation can only improve maize cold tolerance marginally, by only a few degrees Celsius. While there is a wide variation of response to cold acclimation in maize, it does not resemble the capabilities of cold resistant plants like *Arabidopsis Thaliana* (Sobkowiak, 2014). However, the same research group later discovered a mutant line of maize capable of withstanding temperature as low as zero degrees Celsius. This shows how difficult it is to study cold acclimation in maize as its capabilities as a species spread into both extremely susceptible and extremely tolerant.

Stress response in B73 maize alone is highly variable. Plants exposed to identical stresses produced a range of responses. This lead us to question the consistency of our methods. However, image analysis and chlorophyll concentrations are highly correlated (Fig. 13) as they both accurately measured the same stress responses. Validating our methods allows us to conclude that measured variation in this study is a result of biological variation. This makes measuring stress response and drawing conclusions based on those results difficult. With such a variable phenotype, a greater number of replicates might be helpful to determine an accurate measurement of cold stress response. This fact should be considered when planning future experiments.

Correlation Values

	gray	purple	chl A	chl B
green	-0.74	-0.88	0.51	0.59
gray		0.33	-0.57	-0.45
purple			-0.31	-0.51
chl A				0.79

Figure 13: Correlation values between each measurement of greenness (image analysis and chlorophyll concentration).

It was known that humidity was not well controlled for within the growth cabinets. Replicates of the same experiments were conducted throughout different times of year and therefore different seasons. Humidity ranged drastically from 10% to over 70%, enormously effecting typical stress phenotypes. Instead of acquiring burned edges on seedling leaves, many seedlings paled in color when experiencing sub optimal temperatures at high humidity levels (Fig. 14). It is well known that cold stress affects how plants conserve water. Some plants grow deeper root systems (Farooq, 2009), increase stomatal closure (Farooq, 2009) or thicken their cell walls (Sobkowiak, 2016; Farooq, 2009) to conserve water content through durations of cold stress. It can be reasoned that plants, including maize, retain water within their systems as it helps to resist the damages of cold stress. Our observations of exposing seedlings to cold stress at different humidity levels supports this hypothesis.

More replicate plants had been grown in the spring in attempts to combat the wide cold response variation with a larger sample sizes. However, they could not be included in this study due to the high humidity and lack of measurable cold response. As seen in Figure 14, plants experiencing cold and high humidity do not differ from the control as extremely as plants experiencing cold and low humidity. This limited our study to the 6 replicate plants originally grown in the dry winter season.

With this in mind, future cold stress studies should be conducted with large sample sizes during the same season to avoid differences in humidity. Alternatively, a system for removing the humidity from within the growth chambers could be employed. It is critical that all environmental conditions are controlled.

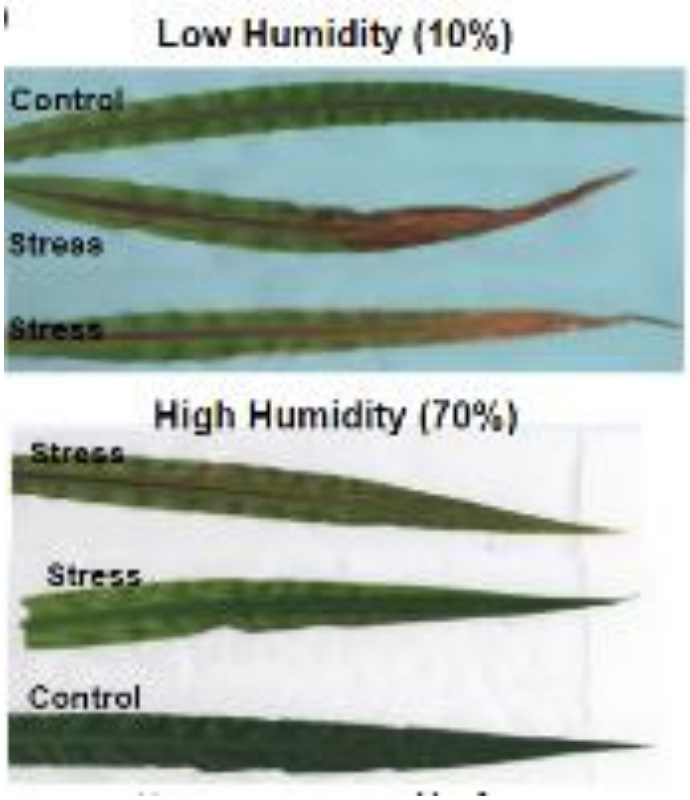


Figure 14: These plants were exposed to identical cold stresses except for humidity conditions. Purpling and burning is near absent in high humidity stress groups, displaying a variable stress response dependent upon humidity.

The purpling effect observed on the cold stressed leaves was discovered to be dependent upon limited nutrients in the soil. Magnesium and phosphorus limitations can produce purpling patterns on seedling leaves (Monsanto Company, 2011). All plants were grown in the same amount of soil but as the priming and control treatments were not stunted by the hard cold stress,

they grew to be much larger than the other two stressed treatment groups. More purpling was observed on these larger plants possibly due to the greater consumption of soil nutrients. Adding Miracle Gro as a supplement prevented any purpling in future experiments. With one less variable to measure, accuracy of our chlorophyll and image analysis methods could increase. Studying plants with proper soil nutrition allows greater focus on actual cold response phenotypes.

RNAseq data revealed the importance of twenty differentially expressed genes in secondary versus primary stress exposure that were also activated by early priming. These "memory" genes are extremely sensitive to cold stress with fold change values above 8.0 or below 0.125. Memory genes have been previously described within maize through dehydration stress as genes with differing transcriptional response from initial to secondary stresses. Memory drought genes included many transcription factors possibly controlling the complex stress memory phenotype through multiple signaling pathways (Ding, 2014). While many transcription factors have been identified to be differentially expressed in response to cold (Thomashow, 1999; Chinnusamy, 2007; Waters, 2016), the memory transcription factors are especially interesting as they initiate a new transcriptional setting responsive to continued cold stress exposures. They potentially alter the cell in ways that provide greater cold tolerance.

Within the twenty identified genes several gene regulation mechanisms were identified. Three transcription factors were all down regulated in response to cold, two protein kinases were affected by cold exposure and one methyltransferase was also down regulated. This allows one to imagine that the gene expression machinery is being altered resulting in a different combination of expressed genes within previously cold stressed plants. At this point, all that can

be done is speculation. Further evidence is required before these genes can be deemed responsible for controlling stress memory.

Mutants containing disruptive transposons are easily obtained from publicly available research collections. Studies conducted with mutant maize lines allow direct evidence towards a gene's effect on a particular phenotype. As has been done with six previously identified cold tolerance candidate genes, mutants could be ordered and planted to provide a population for future research. The outcome of cold stress studies with these mutant populations will confirm the role of these genes in regulating cold memory.

The confirmation of cold tolerance genes could lead to research in manipulation of these genes to produce a cold tolerant strain of maize. This new variety could be engineered through marker assisted breeding programs by targeting DNA markers surrounding the candidate genes. A maize seedling capable of withstanding sub optimal temperatures could vastly lengthen the growing season and potentially produce larger yields. Cold tolerant seedlings could also be grown in regions too cold for traditional maize increasing cultivatable land and thus yield.

Part II: QTL Analysis

Background

To achieve improved performance of agricultural plants such as maize, breeding programs assign a high priority to characterizing traits such as yield, quality and hardiness. Unfortunately, these traits are not genetically simple and therefore make traditional breeding a slow, inefficient guessing game. Such complex traits are controlled by multiple genes in many locations in the genome and are sometimes called multifactorial, polygenic or quantitative. In attempts to identify the many genetic positions that may contribute to a desirable trait, quantitative trait loci analyses were developed.

The purpose of this analysis is to relate phenotypic traits to locations on the genome. This is done using a library of DNA markers. These markers can occur anywhere in the genome; they are where point mutations, insertions, deletions and other replication errors within the DNA occur (Collard, 2005). Libraries of these markers are designed to spread as evenly as possible across chromosomes creating a linkage map. By relating a measurable trait like yield or hardiness to a location on the genome flagged by DNA markers, marker assisted selective breeding can be used to eliminate the guess work of traditional breeding to produce plant breeds with heightened desirable traits.

Quantitative trait analyses are performed on specific populations of plants. Usually, the offspring of two different inbred lines are used. In this study, the recombinant inbred population of intermated B73 and Mo17 (IBM) was used. This population is ideal for quantitative trait studies as its linkage map is well defined allowing “high resolution” analyses to be performed (Collard, 2005). The IBM population was bred to produce homozygous lines each containing a

specific combination of chromosomal regions from their original parents (Collard, 2005). This barcoding effect (Fig. 15) allows for the association of a measurable phenotype to a location on the genome by comparing plants with similar phenotypes and identifying shared chromosomal regions. These regions become known as quantitative trait loci (QTL) and are suspected to contain genes that contribute to the complex characteristic of interest.

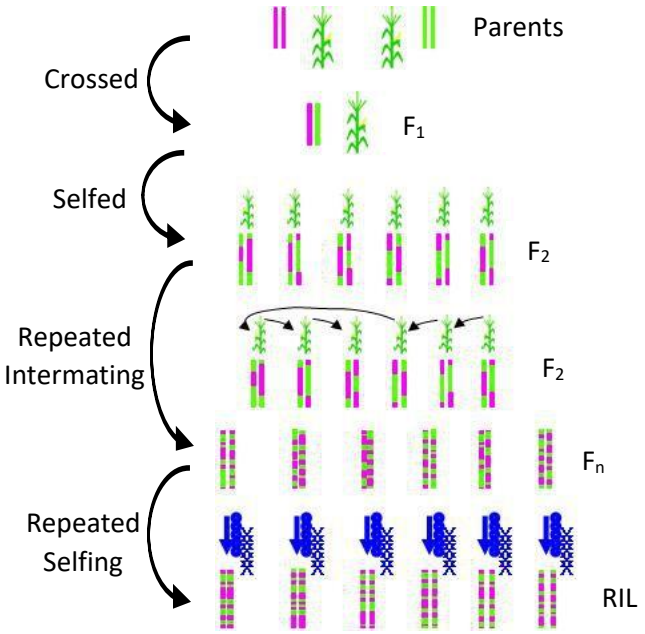


Figure 15: This image explains the creation of the IBM maize population. Image modified from Lee, 2002.

The parental maize lines are cold susceptible (B73) and cold resistant (Mo17) making the IBM population ideal for QTL analyses of cold tolerance. Because of their difference in cold resistance, comparison may reveal genes vital to the maize cold response mechanism. Several methods were used to measure cold resistance in IBM population seedlings. The phenotypes collected after stressing were chlorophyll concentration and percent green calculated from an image analysis. This phenotypic data was used to identify regions on maize chromosomes with genes that contribute to cold resistance. QTLs identified in this study were expected to align with a previous QTL analysis with the same population using a three-point qualitative scale as its cold

tolerance phenotype. Using the known cold tolerance associated regions of the maize genome from the QTLs, gene lists from the public database were obtained for comparison to an RNAseq analysis with plants of similar age exposed to a similar cold stress. The differentially expressed genes in response to cold stress within the chromosomal regions of cold tolerant associated QTLs will reveal candidate cold tolerant genes for future experimentation.

Materials and Methods

QTL parameters and cold exposure

Three replicates of ninety-seven lines from the IBM recombinant inbred population were planted and allowed to grow under greenhouse conditions (74 °F average temp, 15.5/8.5 hour day/night cycles) for 14 days before being cold stressed for 8 hours at 4 degrees Celsius. The plants remained well watered and were provided miracle grow as a soil supplement. Only one third of the IBM lines (97 lines total) were utilized due to limited time and resources.

Measuring cold response

A visual three-point scale of stress (Fig. 16) was assigned to each plant as well as conducting a quantitative Image analysis (Fig. 3) and chlorophyll concentration (Fig. 4) was measured according to the methods in the priming materials and methods.

1) Healthy, solid green leaf (Mo17 like response)
2) Leaf with slight cold damage (Intermediate response)
3) Leaf with obvious cold damage (B73 like response)

Figure 16: Examples of the visual three-point scale of stress on leaves that underwent an 8 hour 4°C cold stress.

QTL analysis

The cold response phenotypes were used to identify four regions of high association on chromosomes 1, 3, 5, and 7. Presence of QTLs were calculated using the package qtl in the statistical program R. Requirements of a QTL were to have a log of odds (LOD) score greater than 3, this confirms a statistically significant association to the QTL region. QTLs identified by more than one phenotype were combined to include the complete region of association with cold response.

Differentially expressed genes

Heat maps were constructed using gene lists obtained from Maizegdb.org spanning the QTL regions merged with the Abiotic Stress RNAseq data collected in 2014 (Figure 17). This ensured only the differentially expressed genes were included from the QTL regions. Research students from Summer 2014 collected 3rd leaf tissue samples from B73 and Mo17 maize plants 2 hours into either control or cold stress conditions. These samples were sent to be RNA-sequenced and were analyzed using DEseq in R. Noise was defined as less than 2 counts per total million and was disposed of (Frey, 2015). Differentially expressed genes were defined as having a Log2 fold change in expression greater than 2 ($|log2| < 2$).

	B73	Mo17
Cold	3 Replicates	3 Replicates
Control	3 Replicates	3 Replicates

Figure 17: Treatment groups sent for RNAseq analysis by the 2014 student researchers. Four different samples consisting of control and cold temperature treatments on different 2 week old maize line seedlings were compared to identify candidate genes for cold tolerance.

Identification of candidate genes

The heat maps were farther analyzed to identify genes behaving uniquely between the B73 and Mo17 maize lines. This was calculated by comparing the amount of up or down regulation between the control and cold treatment genes. Genes were considered to have different patterns of expression If one line remained unchanged while the other changed by an absolute value of fold change greater than 2. Another case of differing patterns of expression occurred when one line was upregulated by a fold change greater than 2 and the other was down regulated as defined by a fold change less than -2.

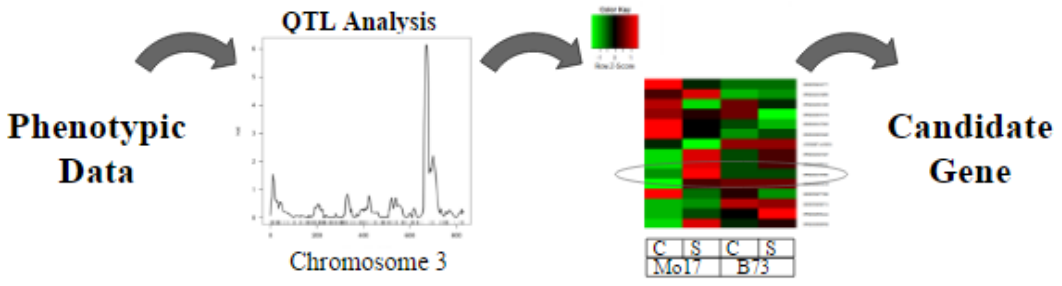


Figure 18: Method of Analysis that identified genes which would be analyzed for differing sequence between B73 and Mo17 variants.

Variant effect predictor

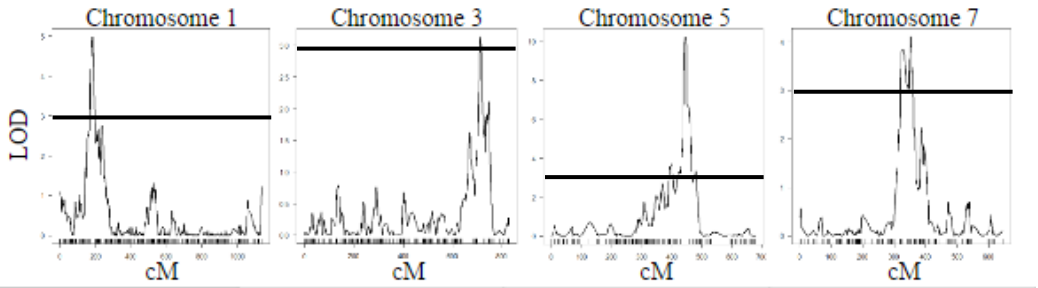
Variation in genes between B73 and Mo17 are frequently single nucleotide polymorphisms (SNPs), insertions or deletions. Lists of these variations were obtained for each candidate gene from the Maizegdb browser track called Mo17 SNPs and Indels (Xin 2013). Consequences for these variations were computed by Gramene's variant effect predictor (McLaren, 2016). Genes containing frameshift mutations or stop mutations were given a consequence rating of "High". Those with missense mutations were assigned to "Moderate" consequence and those with synonymous or intron variants were given a "Low" consequence rating. High consequence mutations were assumed to influence the protein product allowing us to infer that the gene functionally differs between the two lines possibly resulting in the differing tolerance to cold stress.

Results

Approximately one third of the IBM population was selected randomly to save resources and time. Ninety-five lines from the IBM maize population were grown in triplicate along with the parental lines (B73 and Mo17). After 14 days of growth at greenhouse conditions, seedlings were exposed to a 4°C cold stress for 8 hours. Two days after the cold stress, response was measured through a visually determined 3 point scale, image analysis and chlorophyll concentration. Using these measurements, cold response was associated with four regions on the genome using the qtl R package. This is done by identifying IBM lines that behave significantly different from the rest of the population and identifying chromosomal regions that are shared by those lines. This is why the barcoding effect seen in the IBM population is so valuable. These regions of association are called quantitative trait loci (QTL) and were discovered upon chromosome 1, 3, 5, and 7. The QTL on chromosome 7 is larger than the other three QTLs

combined. It is largest in length and gene composition. However, QTL 5 was most significantly correlated with phenotype, exhibiting an LOD score greater than 10 (Table 2).

Table 2: Identified QTL characteristics and locations. Statistically significant QTL regions are defined as having peaks with an LOD score greater than three.

QTL plot				
Methods that identified the QTL	3 pt scale	Chlorophyll A Concentration, Chlorophyll B Concentration, Image Analysis,	3 pt scale	Chlorophyll B Concentration, Abs at 645nm (Chlr B)
Length of QTL (bp)	23,677,730-27,398,959 bp	209,705,866-222,439,054 bp	183,069,354-191,229,973 bp	131,984,321-163,210,280 bp
Length of QTL (cM)	150-235cM	600-762cM	440-460cM	320-471cM
Genes in QTL	210 genes	920 genes	532 genes	2178 genes

The QTLs in the above table span chromosome regions containing multiple overlapping QTLs from the same location. A single QTL is determined by a single phenotype. Figure 19 shows each individual QTL color coded with the phenotype it coresponds to and its location on the chromosme. Some QTLs are very small while others are much larger. Lines across each chromosome represent DNA marker density. Regions without markers lack data for association so QTLs frequently span across regions of low DNA marker density. While some lines within

the IBM population were not utilized in this study, every DNA marker from the IBM population was utilized in the analyses.

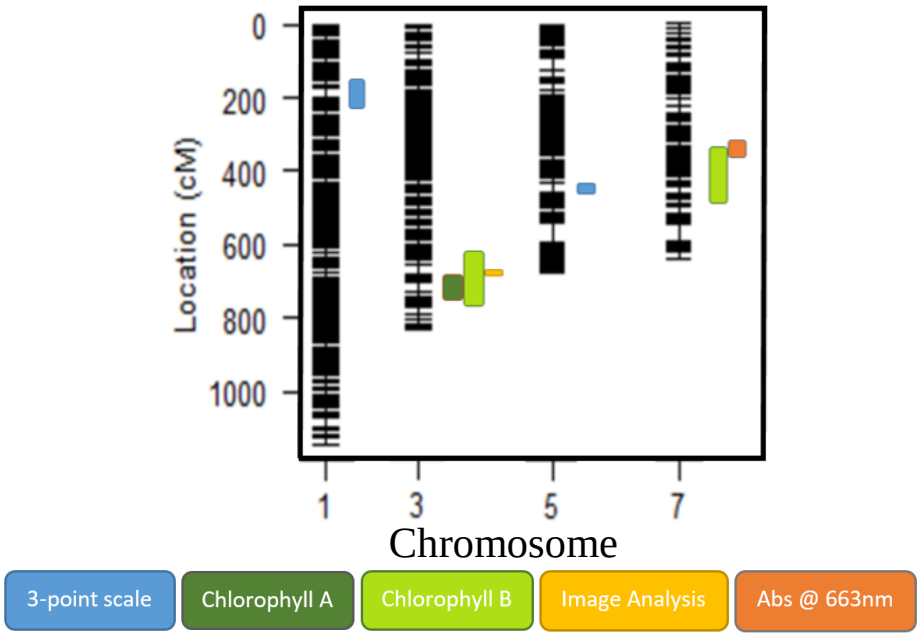
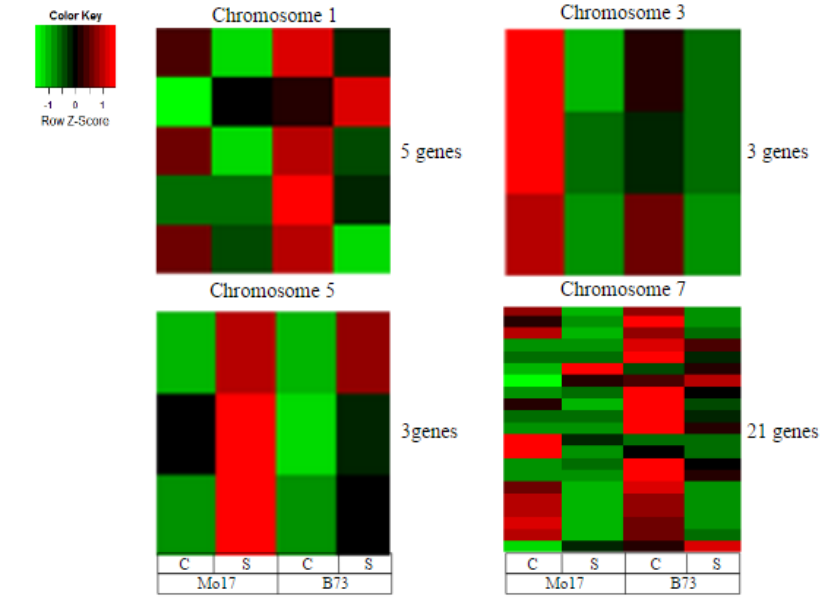


Figure 19: Linkage map of QTLs identified on chromosomes 1, 3, 5 and 7 as described by different cold tolerance phenotypes. Black bars across chromosomes indicate presence of molecular markers along the centimorgan position of the chromosome.

Once QTLs were discovered, four areas of the maize genome could be focused on for gene expression studies. Gene lists were constructed containing every gene within the QTL regions. Gene expression data from an RNAseq study comparing the cold response between B73 and Mo17 was used to identify cold responsive genes from within the QTL gene lists. Cold responsive genes were defined as genes differentially expressed (DE) by a log2 fold change greater than 2 when comparing stressed samples to control samples. At this point, a candidate gene list contained 461 genes.

More interesting cold responsive genes were identified by comparing the nature of the cold responsive genes in B73 and Mo17. Genes with unique cold response were defined as genes

that were either activated in one parental line but off in the other, or genes that were upregulated in one parental line but down regulated in the other. This identified 32 candidate cold responsive genes that differed in the nature of their expression in the two parental lines.



	DE genes	(↑,↓) genes	(↑,=) or (↓,=) genes	candidate genes
QTL1	34	1	4	5
QTL3	102	0	3	3
QTL5	60	0	3	3
QTL7	265	2	19	21

Figure 20: Heatmaps of significantly differentially expressed genes within the QTL that behave differently in B73 and Mo17. These are the candidate genes before undergoing sequence comparison analyses.

These genes within the QTL regions have unique cold response expressions when comparing B73 and Mo17. It was farther speculated that they may differ in sequence as well. Sequences of the candidate genes from B73 and Mo17 genomes were entered into the Gramene variant effect predictor which calculates the effect of sequence variation. Sequence mutations can receive a high, moderate or low consequence rating. These ratings relate to the likelihood of the protein product being altered in function by the mutations.

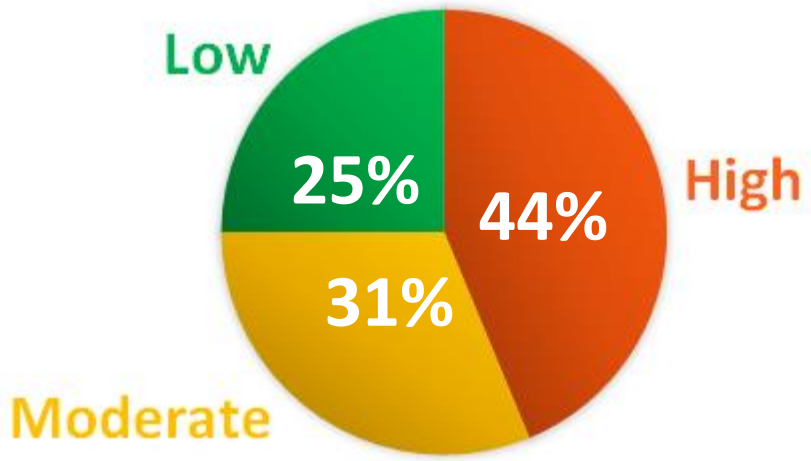


Figure 21: Mutation consequence distribution in candidate genes. “High” consequence includes frameshift mutations or stop mutations, “Moderate” consequence reports missense mutations, “Low” consequence includes synonymous or intron variants.

The final list of candidate cold tolerance genes with sequence alterations resulting in high consequence mutations can be seen in Table 3. The location, type of mutation present between B73 and Mo17 is listed along with the function or classification of the gene.

Table 3: Final 13 candidate genes, location, mutation type and function.

Gene ID	Chr	Mutation type	Function
AC208897.3_FG003	1	Stop, Frame Shift	Hypothetical protein/Cinnamyl alcohol dehydrogenase
GRMZM2G153799	1	Frame Shift	Spermine oxidase
GRMZM2G024391	3	Frame Shift	Hypothetical Membrane Protein
GRMZM2G319955	3	Frame Shift	Hypothetical Protein
GRMZM2G315140	5	Stop, Frame Shift	Hypothetical DNA binding Protein
GRMZM5G862799	7	Frame Shift	Hypothetical F Box containing Protein
GRMZM2G841684	7	Stop, Frame Shift	Hypothetical Protein
GRMZM2G006505	7	Frame Shift	Receptor kinase isolog
GRMZM2G379780	7	Stop, Frame Shift	Receptor kinase
GRMZM2G325783	7	Stop, Frame Shift	Hypothetical Protein
GRMZM5G871418	7	Stop, Frame Shift	Hypothetical Protein
GRMZM2G161809	7	Frame Shift	Transparent Testa 12 Protein
GRMZM2G041381	7	Stop	Histone H2A

Discussion:

Four QTL regions were discovered to be associated with cold tolerance phenotypes. Through evaluation of the genes within these QTLs, thirteen candidate genes were identified as cold tolerant genes. Farther research could reveal these genes being vital to marker assisted breeding programs with intentions of producing a cold tolerant maize line.

Previous QTL studies on cold stress in maize have identified QTL regions on chromosomes 4, 5, 6, 7 and 9 (Hu, 2016) when measuring germination of seeds in sub optimal temperatures, chromosomes 3 and 6 (Rodriguez, 2008) when measuring leaf color and a review of 4 QTL studies measuring various cold responsive phenotypes identified QTL regions on all ten maize chromosomes (Sobkowiak, 2014). Cold responsive genes are located across the maize genome with no centralized location identified thus far. While QTL regions may seem excessively abundant with little consistency, they provide areas of focused research. Studying any portion of the maize genome is easier than studying the entire genome.

It must be noted that the QTL analysis conducted in this study is not as powerful as it could be. Due to time, resource and material restrictions, only 97 of the 300 IBM population lines were tested for cold stress phenotypes. This produced multiple QTLs that likely could be refined. These multiple QTLs were discovered using several types of cold tolerance phenotypes. While it is possible that phenotypic data had differing statistical power revealing more or less of a finite number of cold tolerance QTLs, it is more likely that the QTLs discovered with certain phenotypic data are more related to the phenotypic test. For example, chlorophyll concentration may reveal QTLs containing chlorophyll synthesis enzymes whereas image analysis data could reveal osmotic regulating genes evident in dry browning regions of leaf. More cold response phenotypes could be added to the analysis like growth rate or yield to identify more diverse QTL

regions. Power of QTL analyses could be increased by using all 300 IBM lines with a greater number of replicates and phenotypic measurements. Such an analysis could elucidate the true nature of the discovered QTLs in this study.

Identified among the 13 candidate genes are two protein kinases and two proteins containing DNA binding motifs. These functions suggest regulation of gene expression. Farther analysis is required however, it is possible these genes could be creating a stress tolerant state for the cells through gene expression regulation. Many of the candidate genes have unknown function and have yet to be studied. This is also exciting as there is only new knowledge to gain of these genes.

Comparison of gene expression between two maize lines can reveal candidate genes responsible for phenotypic differences in cold tolerance when paired with a quantitative trait locus analysis. Natural genetic biological variation between the lines can also explain differences in how the lines respond to stress. In this analysis, 13 candidate genes potentially important for tolerance to early cold stress response were identified. These genes fall within cold tolerance QTL regions, are differentially expressed in response to cold, behave differently in cold responsive and tolerant lines and contain sequence variation between B73 and Mo17 likely resulting in altered gene function.

Conclusion:

The goal of this project was to develop quantitative methods for measuring cold stress response in maize seedlings, characterize the phenotypic and transcriptomic response to repeated cold stress exposure and to identify candidate cold tolerance genes within the determined QTL regions. Our phenotypic data showed no evidence for the positive effect of priming to cold stress

in maize seedlings. However, we had more success identifying candidate cold tolerance genes using RNAseq and QTL analyses.

Thirty-three candidate genes for cold tolerance in maize were discovered using two lines of thought involving methods like RNAseq, QTL analysis, phenotypic evaluation and sequence comparisons. The importance of these genes must be farther confirmed by obtaining direct evidence through studying mutant lines for each gene. This process has begun for the initial six genes identified in 2015. Mutant lines have been obtained, genotyped and grown up in the field to produce more seeds for future experimentation.

Once confirmed as cold tolerance genes, manipulations of maize lines to express or silence these genes could greatly benefit performance of seedlings in sub optimal temperatures. Seedlings with greater resistance to cool temperatures would push the range of farmable land into the extremes. Maize of this variety could be grown farther from the equator and at higher altitudes. The growing season could begin earlier without fear of late frosts. The impact of such a robust line of maize could facilitate the increased production of feed, food and fuel making modern lifestyles more sustainable.

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